substantially compromising the effectiveness of the produced SIA in glucose uptake and insulin receptor binding activities. For example, several different amino acid residues may be added or taken off at either end without substantially decreasing the activity of the produced SIA. In addition, the amino acid Gly may be replaced with any amino acid residue.

Please replace the paragraph bridging pages 13 and 14 with the following new paragraph:

EXAMPLES

EXAMPLE 1 - MATERIALS AND METHODS

Cloning and expression of single chain insulin analog (SIA) DNA in *E. coli.*

SIA-1 DNA encoding Gly-Gly-Gly-Pro-Gly-Lys-Arg sequence in the linker region of SIA was generated by polymerase chain reaction (PCR) using five overlapping oligonucleotides of 65-68 bases in length. The SIA-1 DNA was constructed by considering the codon usage of *E. coli* to increase the expression level of SIA in the bacterial hosts. The resulting SIA-1 DNA sequence was:
ATG/TTC/GTT/AAT/CAG/CAC/CTG/TGC/GGC/TCT/CAC/CTG/GTA/GAA/GCT/CT
G/TAC/CTG/GTT/TGC/GGT/GAA/CGT/GGT/TTT/TTC/TAC/ACC/CCG/AAA/ACC/
GGT/GGT/GGT/CCG/GGT/AAA/CGT/GGC/ATC/GTT/GAA/CAA/TGC/TGT/ACT/A
GC/ATC/TGC/TCT/CTC/TAC/CAG/CTG/GAG/AAC/TAT/TGT/AAC/TAG/TAA
(SEQ ID NO:3). The N-terminal pentapeptide sequence (PSDKP) of TNF-α was used as a fusion partner to produce SIA-1 with high-level expression in *E. coli.* For convenience in the purification process, 10 histidine residues and a methionine residue for chemical cleavage were inserted between the PSDKP sequence and SIA-1. A DNA fragment encoding the PSDKP sequence and 10 histidine residues was chemically synthesized. After digestion with restriction endonucleases *Nde*I and *Bam*HI, the DNA fragment was inserted downstream of the T7 promoter of the expression plasmid pET-3a, which was linearized with the same restriction endonucleases, and the resulting plasmid was named pET. The gene encoding SIA-1 was digested with *Bam*HI and *Hin*dIII and inserted into the *Bam*HI and *Hin*dIII enzyme restriction sites of the pET plasmid and the resulting plasmid was named pET-SIA-1. This expression plasmid was then used to transform *E.*

coli BL21 (DE3) cells, and the fused single chain insulin analog was expressed as inclusion bodies. The inclusion bodies of the fusion protein were sulfonated at their cysteine residues and chemically cleaved by CNBr treatment. S-sulfonated SIA-1 was purified by cation-exchange chromatography (Pharmacia Biotechnology) and refolded by addition of β-mercaptoethanol and analyzed by analytical reverse-phase HPLC. Briefly, sulfonated SIA-1 (0.37 mg/ml) was converted to SIA-1 with native disulfide pairings in 50 mM glycine buffer, pH 11.0, at 4°C using 2 equivalents of β-mercaptoethanol. After 20 hr, the protein solution was acidified to pH 2.5 to terminate the reaction, loaded onto a Zorbax C8 column and eluted with a linear gradient of 90% acetonitrile. The fractions containing the desired material were pooled, frozen and lyophilized.

a2 cont

Please replace the first full paragraph at page 14 with the following new paragraph:

Other SIA DNAs encoding Arg-Arg-Gly-Pro-Gly-Gly-Gly, Gly-Gly-Gly-Gly-Gly-Lys-Arg (SEQ ID NO:4), Arg-Arg-Gly-Gly-Gly-Gly-Gly (SEQ ID NO:5), Gly-Gly-Ala-Pro-Gly-Asp-Val-Lys-Arg (SEQ ID NO:6), Arg-Arg-Ala-Pro-Gly-Asp-Val-Gly-Gly (SEQ ID NO:7), Gly-Gly-Tyr-Pro-Gly-Asp-Val-Lys-Arg (SEQ ID NO:8), Arg-Arg-Tyr-Pro-Gly-Asp-Val-Gly-Gly (SEQ ID NO:9), Gly-Gly-His-Pro-Gly-Asp-Val-Lys-Arg (SEQ ID NO:10) and Arg-Arg-His-Pro-Gly-Asp-Val-Gly-Gly (SEQ ID NO:11) sequence in the linker region of SIA, respectively, were prepared by PCR using the SIA-1 gene as a template DNA. These genes were digested with *Bam*HI and *Hin*dIII and inserted into plasmid pET. The resulting plamids were named pET-SIA-2, pET-SIA-3, pET-SIA-4, pET-SIA-5, pET-SIA-6, pET-SIA-7, pET-SIA-8 and pET-SIA-9, respectively. These expression plasmids were then used to transform *E. coli* BL21 (DE3) cells, and the fused SIAs were expressed as inclusion bodies. The purification processes for SIAs were essentially the same as that of SIA-1.

a3 ✓

Please replace the paragraph bridging pages 15 and 16 with the following new paragraph:

Construction of pSIA and pLPK-SIA

a4 ✓

The SIA cDNA from pET-SIA was subcloned into the PCR-script sk^+ (Invitrogen, San Diego, CA) at the BamHI/HindIII site. Then the SV40 poly(A) signal sequence from pCDM8 (Invitrogen) and the SV40 enhancer from the pGL3 (Promega, Madison, WI)

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were amplified by PCR and subcloned at the HindIII/ApaI and ApaI sites, respectively. The albumin leader sequence (72 base pairs) was inserted in front of the SIA cDNA using a ExSite™ PCR-based Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the following primer set: the 5' primer (63 mer) containing a sequence complementary to the upstream 27 nucleotides of the SIA cDNA and the 5' 36 nucleotides of the human albumin leader sequence (TTAGCTCGGC TTATTCCAGG GGTGTGTTTC GTCGAGATTT CGTTAATCAG CACCTGTGCG GCT (SEQ ID NO:12)) and the 3' primer (63 mer) containing the 3' 36 nucleotides of the albumin leader sequence and the 27 nucleotides of the SIA cDNA (AGAGAAAAAG AAGGGAAATA AAGGTTACCC ACTTCATGGA TCCGCCCAGT CGTCGACGCT GCT (SEQ ID NO:13)). The clone containing the albumin leader sequence was isolated and designated as pSIA. The final construct, pLPK-SIA, was generated by insertion of the promoter of the rat LPK gene (-3193 to +18) amplified by PCR into pSIA at the XbaI/SalI site.

a4 cont

Please replace the second full paragraph at page 16 with the following new paragraph:

PCR and RT-PCR analysis.

To examine the presence of SIA DNA in plasmid-injected rats, PCR was performed using the sense primers derived from the T7 or LPK promoter region (5' GTAATACGACTCACTATAG GGC 3' (SEQ ID NO:14) for pSIA-injected rats; 5' ATTTCGAATAAGAAGAGGAAGGGAAG 3' (SEQ ID NO:15) for pLPK-SIA-injected rats) and the antisense primers derived from the 3' terminus of the SIA gene (5' GCGCAAGCTTTTACTAGTTACAATAGTT 3' (SEQ ID NO:16). To detect SIA mRNA, the total RNA was isolated form various tissues and RT-PCR was performed using the primers 5' GCGCGGATCCATGTTCGTTAATCAGCAC 3' (SEQ ID NO:17) and 5' GCGCAAGCTTTTACTAGTTACAATAGTT 3' (SEQ ID NO:18). β-actin mRNA was amplified as an internal control.

✓ a5